REMARKS

Claims 27-34 are pending.

Applicants respectfully traverse the present rejections.

Calculation of Extension Periods:

Applicants have submitted herewith a Request for Continued Examination requesting continued examination of the above-identified application in view of the Amendment and Response mailed May 7, 2007 and in view of the following remarks and arguments. Along with Applicants' Request for Continued Examination, Applicants have submitted herewith a petition for extension of time requesting a two-month extension of time. In the Advisory action mailed June 20, 2007, it is indicated that the period for reply expires 3 months from the mailing date of the final rejection. Applicants believe this is in error as Applicants submitted an Amendment and Response within 2 months of the mail date of the final rejection. MPEP 706.07(f). Specifically, the final rejection was mailed March 7, 2007 and Applicants' Amendment and Response was mailed May 7, 2007. For the Examiner's convenience, Applicants have included the following documents, which evidence these dates: a copy of the first page of the Final Office action indicating it was mailed March 7, 2007, a copy of the transmittal sent with the Amendment and Response indicating it was mailed May 7, 2007, and a copy of the first page of the Amendment and Response indicating it was mailed May 7, 2007.

Thus, for purposes of calculating extension fees, Applicants respectfully submit that a petition for a 2-month extension of time is due. A petition for a 2-month extension of time is filed herewith. In case any of Applicants' calculations are in error, Applicants have noted in the Request for Continued Examination, the transmittal letter, and the petition for extension of time that the Commissioner may charge payment of any additional fees to Deposit Account No. 23-1925.

35 U.S.C. § 101

Claims 27-34 remain rejected under 35 U.S.C. §101 as allegedly not supported by either an asserted utility that is specific and substantial or by a well-established utility.

Applicants thank the Examiner for acknowledging that mRNA levels are predictive of polypeptide levels. In that regard, Applicants understand that the following references have been overcome and are no longer being relied upon to support the rejections: Chen et al., Hu et al., Haynes et al., Gygi et al., Lian et al., Fessler et al., Greenbaum et al., Nagaraja et al., Waghray et al., Sagnaliev et al., Lilley et al., King et al., Bork et al., and Madoz-Gurpide et al.

According to the Advisory action, the basis of the maintained rejection is "solely that gene amplification levels (genomic DNA levels) are not predictive of mRNA or polypeptide levels." Page 2 of the Advisory action mailed June 20, 2007. In maintaining rejection of the claims on this basis, the Advisory action acknowledges Applicants' argument that similar claims supported by the same utility based on similar data have been allowed in U.S. Patent No. 7,208,308 but does not find this persuasive because the actions of one examiner are not binding on another. The Advisory action also states that the Examiner has considered Orntoft, Hyman, and Pollack anew but maintains that these references are not sufficient to overcome the rejection. In maintaining the rejection, the Advisory action relies on Godbout, Sen and Pennica.

Applicants respectfully disagree with the maintained rejection of claims 27-34. Specifically, while Applicants acknowledge that the actions of one Examiner are not binding upon another, Applicants maintain that allowance of similar claims based on similar assertions of utility that rely on similar data is persuasive evidence that Applicants' asserted utility does not violate any scientific principle nor is it wholly inconsistent with contemporary knowledge in the art. A rejection for lack of utility is only proper when the asserted utility violates a scientific principle or is wholly inconsistent with contemporary knowledge in the art. *See* MPEP § 2107.02 III B, citing *In re Gazave,* 379 F.2d 973 (CCPA 1967). Clearly, that is not the case here. Indeed, the PTO has acknowledged on

more than one occasion that a utility such as that asserted herein is sufficient. *See e.g.,* U.S. Patent Nos. 7,208,308, US Patent App. Ser. Nos. 10/123,214 (Notice of allowance mailed 6/14/07); 10/131,825 (Notice of allowance mailed 6/18/07); 10/131,813 (Notice of allowance mailed 5/29/07); 10/140,863 (Notice of allowance mailed 6/21/07); 10/140,923 (Notice of allowance mailed 6/8/07); 10/141,755 (Notice of allowance mailed 6/20/07); 10/141,698 (Notice of allowance mailed 6/14/07); 10/142,762 (Notice of allowance mailed 6/14/07); 10/143,113 (Notice of allowance mailed 6/12/07); 10/230,417 (Notice of allowance mailed 5/30/07); 10/181,000 (Notice of allowance mailed 6/7/07); 10/184,627 (Notice of allowance mailed 6/21/07); 10/187,886 (Notice of allowance mailed 6/21/07); 10/187,739 (Notice of allowance mailed 6/21/07); and 10/219,077 (Notice of allowance mailed 6/18/07). Issuance of these 16 patents, which were examined by at least 10 different examiners (one of whom is the Examiner of the present application), is direct evidence that Applicants' assertion of utility satisfies the requirements of 35 U.S.C. § 101.

Further, Applicants have provided persuasive evidence that the gene amplification demonstrated in the present application (approximately 87% of lung tumor tissues and approximately 53% of colon tumor tissues listed in Table 9 and tested showed greater than 2-fold DNA amplification) is art recognized to correlate with mRNA levels and polypeptide levels. Specifically, Applicants maintain that Orntoft, Pollack, and Hyman demonstrate that gene amplification levels are more likely than not to correlate with mRNA levels. In addition, Applicants herein cite and rely on 3 additional references that demonstrate the art accepts that *more often than not*, which is the standard that must be satisfied for utility, there is good correlation between gene amplification and mRNA levels.

Lin *et al.,* "Expression Cloning of Human EGF Receptor Complementary DNA: Gene Amplification and Three Related Messenger RNA Products in A431 Cells." *Science,* 1984. 224(4651):843-848 (Abstract submitted herewith) teaches:

> Over-expression of EGF receptors in A431 cell lines correlates with increased EGF receptor mRNA levels and amplification (up to 110 times) of the apparently singular EGF receptor gene.

Imam *et al.,* "Analysis of Thymidylate Synthase Gene Amplification and of mRNA leves in the Cell Cycle." *J. Biol. Chem.* 1987. 262(15):7368-7373 (submitted herewith) teaches that the increase in gene copy number for thymidylate synthase is reflected in elevation of thymidylate synthase mRNA levels. More specifically, Imam *et al.* report:

> [T]he amplification of the gene for thymidylate synthase in the murine cell line L1210:C15. . . . Direct analysis of the TS gene by Southern blotting showed that the gene for thymidylate synthase has amplified 40- to 50- fold in the resistant cell line compared to the parental cell line and Northern analysis showed that there was a concomitant 40- to 50- fold elevation of thymidylate synthase mRNA levels.

Blancato *et al.,* "Correlation of amplification and overexpression of the *c-myc* oncogene in high-grade breast cancer: FISH, *in situ* hybridization and immunohistochemical analyses." *Brit. J. Cancer,* 2004. 90:1612-1619 (submitted herewith), analyzed gene amplification, RNA expression, and protein expression of the c-*myc* gene on archival tissue specimens of high-grade human breast cancer. The specific question addressed by Blancato was "whether expression of c-*Myc* mRNA and protein were correlated with its gene copy amplification." Abstract. In answering this question, Blancato *et al.* report:

> Statistically significant correlations were identified among the gene amplification indices, the RNA expression scores and the protein expression scores. *C-myc* gene amplification, as detected by FISH, was significantly associated with expression of its mRNA, as measured by the intensity of *in situ* hybridization in invasive cells (P=0.0067), and by the percentage of invasive cells positive for mRNA expression (*P*=0.0006). C-*myc* gene amplification was also correlated with the percentage of tumour cells which expressed high levels of its protein, as detected by immunohistochemistry in invasive cells (*P*=0.0016). Thus, although multiple mechanisms are known to regulate normal and aberrant expression of c-*myc*, in this study, where *in situ* methodologies were used to evaluate high-grade

> human breast cancers, *gene amplification of c-myc appears to play a key role in regulating expression of its mRNA and protein*.

(Emphasis added).

Thus, Applicants respectfully submit that these references, taken in combination with the references previously discussed and of record, and with the evidence of the PTO issuing at least 16 patents with similar claims based on similar assertions of utility relying on similar data, demonstrate that *in general*, it is more likely than not that gene amplification will correlate with mRNA and/or polypeptide overexpression. Thus, it is more likely than not that the claimed polypeptide, which is encoded by a nucleic acid that is amplified in lung and colon tumors, is overexpressed and useful as a diagnostic.

According to the Advisory action, "[t]he general concept of gene amplification's lack of correlation with mRNA /protein overexpression was addressed with reference to Sen in the Office Action mailed 24 March 2003. Specifically, cancerous tissue is known to be aneuploid, that is, having an abnormal number of chromosomes (see Sen, 2000, Curr. Opin. Oncol. 12:82-88). The data presented in the specification were not corrected for aneuploidy. A slight amplification of a gene does not necessarily correlate with oxerexpression in a cancer tissue, but can merely be an indication that the cancer tissue is aneuploid." Page 4 of the Advisory action mailed June 20, 2007.

Applicants respectfully disagree with this rejection and respectfully submit that this ground of rejection was previously overcome. Specifically, as stated in Applicants' response mailed June 24, 2003:

> The data presented in the specification are from experiments using appropriate controls for aneuploidy (see, for example, page 137, lines 13-16). Applicant used framework mapping to control for aneuploidy and to ensure that the observed ΔCt data represent relevant gene amplification. Thus, the reported data are an indication of relevant gene amplification, and support the conclusion that

PRO347, and related proteins and antibodies, can be used as a cancer diagnostic.

In response to this, according to the Office action mailed September 24, 2003, the Examiner "concede[d] . . . that proper controls for aneuploidy were used." Page 6 of the Office action mailed September 24, 2003. Applicants respectfully submit that for these reasons, Sen and this basis for maintaining rejection of the claims is overcome.

The Advisory action also maintains the position that Godbout *et al.* provides evidence that the claims are not supported by an adequate utility because allegedly "there is no evidence that PRO347 confers any growth advantage to a cell, and thus it cannot be presumed that the protein is overexpressed because the gene is amplified." Page 5 of the Advisory action mailed June 20, 2007.

Applicants respectfully disagree. First, Godbout does not teach that amplified genes are *only* overexpressed if they provide a selective advantage. Rather, Godbout, simply states that "it is unlikely that a gene located ~ 400 kb from the *MYCN* gene will be consistently amplified as an intact unit unless its product provides a growth advantage to the cell." Page 21162 of Godbout. Thus, Godbout suggests that selective advantage may play a role in why a particular gene may be co-amplified with another gene. Applicants respectfully submit that this aspect of the Godbout teachings is not relevant to Applicants' assertion of utility, which is not based on any gene that is alleged to be co-amplified. In addition, Godbout does not discuss whether there is a correlation between gene amplification and protein overexpression of a gene that is not co-amplified. Thus, Applicants respectfully maintain that Godbout does not teach that Applicants assertion of utility is wholly inconsistent with or violates any scientific principles nor does Godbout make it more likely than not that one of ordinary skill in the art would doubt Applicants' assertion of utility.

Although the Advisory action does not discuss Pennica in detail, it does mention Pennica as evidence supporting the rejection. Applicants maintain that this reference

does not demonstrate Applicants' asserted utility is inconsistent with or violates any scientific principle. Specifically, Pennica analyzed relative gene amplification and RNA expression of *WISPs-1, 2,* and *3* in cell lines, colorectal tumors, and normal mucosa using quantitative PCR. Pennica noted that *WISPs-1* and *2* had copy numbers that were significantly higher than one, indicating gene amplification. Pennica further noted that the copy number for *WISP-3* was indistinguishable from one, indicating no or minimal gene amplification. Pennica at 14720. Pennica examined the levels of *WISP* transcripts in RNA isolated from 19 adenocarcinomas and their matched normal mucosa using quantitative PCR and found: *WISP-1* RNA levels displayed good correlation to gene amplification of *WISP-1.* Specifically, Pennica found that RNA levels of *WISP-1* in tumor tissue were significantly increased in 84% (16/19) of the human colon tumors examined when compared with normal adjacent mucosa. *See* Pennica at 14721, Figure 7. However, Pennica also found that *WISP-3* RNA levels did not significantly correlate with *WISP-3* gene amplification. In particular, although *WISP-3* did not display significant gene amplification levels, RNA levels in tumor tissue were overexpressed in 63% (12/19) of the human colon tumors examined when compared with normal adjacent mucosa. *See* Pennica at 14721. Further, Pennica reports that *WISP-2* gene amplification levels are negatively correlated with RNA expression levels. That is, although *WISP-2* was significantly amplified, RNA levels of *WISP-2* in tumor tissues were significantly lower than RNA levels of *WISP-2* in normal adjacent mucosa. *See* Pennica at 14721.

Applicants respectfully disagree that these teachings demonstrate that *more likely than not* one of ordinary skill in the art would not expect gene amplification levels to correlate with protein overexpression. First, *WISP-1* gene amplification and RNA expression levels showed a significant positive correlation. Second, although *WISP-3* was not significantly amplified, it was amplified (*P*=1.666) and overexpressed. Third, although *WISP-2* gene amplification and RNA expression levels seemed to be inversely related, Pennica suggests that this result might be inaccurate: "[b]ecause the center of the 20q13 amplicon has not yet been identified, it is possible that the apparent amplification observed for *WISP-2* may be caused by another gene in this amplicon." *See* Pennica at

14722. Thus, because the RNA expression pattern of *WISP-2* cannot be accurately attributed to gene amplification of *WISP-2,* this result should be disregarded. Indeed, the teachings of Gobbout taken with Pennica suggest that Pennica's conclusion that the observed amplification is not actually attributable to *WISP-2* is correct. Moreover, as discussed above, in the present case, appropriate controls for aneuploidy were used and page 137 of the present specification explains the procedures performed to confirm that the observed gene amplification was attributable in the present case to PRO347. Therefore, Pennica *et al.* *does not make it more likely than not* that the present invention is not supported by a specific, substantial, and credible utility.

Applicants understand that the evidence currently under consideration includes references relied on by the Office: Sen, Godbout, and Pennica; and references relied on by Applicants: Orntoft, Hyman, Pollack, Lin, Imam, and Blancato. In addition, Applicants rely on 15 recently allowed patent applications that claim a polypeptide whose diagnostic utility is based on demonstrated gene amplification in cancerous tissues. These allowed applications are direct evidence that in the PTO's view, it is more likely than not that demonstrated gene amplification correlates with protein overexpression.

Section 2107.02 of the MPEP requires the Office to consider whether the *totality of the evidence* submitted regarding the asserted utility demonstrates that the asserted utility violates a scientific principle or is *wholly* inconsistent with contemporary knowledge in the art. For the reasons discussed above, Applicants respectfully maintain that the *totality* of this evidence currently under consideration **does not** demonstrate that the asserted utility violates a scientific principle, nor is the *totality* of the evidence *wholly* inconsistent with contemporary knowledge in the art. Indeed, the totality of the evidence demonstrates that it is more likely than not that PRO347 is overexpressed in lung or colon tumor tissues. For these reasons, Applicants maintain that this rejection is improper and request that it be withdrawn.

35 U.S.C. § 112 ¶ 1, Enablement-Utility

Claims 27-34 stand rejected under 35 U.S.C. § 112 ¶1, because it is alleged that the presently claimed invention is not supported by a substantial utility, and therefore, one skilled in the art would not know how to use the claimed invention. As discussed in the remarks above, Applicants respectfully submit that the claimed polypeptide is supported by a substantial utility. Thus, Applicants respectfully request the Examiner reconsider and withdraw this ground of rejection.

SUMMARY

Applicants believe that currently pending Claims 27-34 are patentable. The Examiner is invited to contact the undersigned attorney for Applicants via telephone if such communication would expedite allowance of this application.

Respectfully submitted,

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1: Science. 1984 May 25;224(4651):843-8.

Science AAAS

Expression cloning of human EGF receptor complementary DNA: gene amplification and three related messenger RNA products in A431 cells.

Lin CR, Chen WS, Kruiger W, Stolarsky LS, Weber W, Evans RM, Verma IM, Gill GN, Rosenfeld MG.

In order to further define the mechanisms by which polypeptide growth factors regulate gene transcription and cellular growth, expression cloning techniques were used to select human

epidermal growth factor (EGF) receptor complementary DNA clones. The EGF 3' coding domain shows striking homology to the transforming gene product of avian erythroblastosis virus (v-erbB). Over-expression of EGF receptors in A431 cell lines correlates with increased EGF receptor mRNA levels and amplification (up to 110 times) of the apparently singular EGF receptor gene. There appear to be three cytoplasmic polyadenylated RNA products of EGF receptor gene expression in A431 cells, one of which contains only 5' (EGF binding domain) sequences and is postulated to encode the secreted EGF receptor-related protein.

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Analysis of Thymidylate Synthase Gene Amplification and of mRNA Levels in the Cell Cycle*

(Received for publication, November 10, 1986)

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We report that the gene for thymidylate synthase (TS) is amplified in the mouse cell line L1210:C15 that was selectively grown in increasing concentrations of the competitive inhibitor of thymidylate synthase, CB3717. The gene is amplified 50-fold compared to the parental cell line. Amplification has not been accompanied by any major rearrangements, and the increase in gene copy number is reflected in elevation of thymidylate synthase mRNA levels. The amplification is relatively stable as there was only a 2- to 3-fold decrease in the number of amplified TS genes when cells were grown in the absence of selection for 375 generations. We also observe a 30- to 40-fold increase in number of copies of the dihydrofolate reductase gene with 7-fold elevation of the RNA product, and we suggest that this may be due to cross-inhibition of dihydrofolate reductase by CB3717.

Thymidylate synthase mRNA levels in L1210 and L1210:C15 show no variation within the different phases of the cell cycle but are significantly reduced during quiescence.

Thymidylate synthase (EC 2.1.1.45) catalyzes the conversion of deoxyuridylic acid (dUMP) to thymidylic acid (TMP) by reductive methylation using N^5,N^{10}-methylene tetrahydrofolate (5,10-CH_2FH_4) as a co-substrate. In proliferating cells that are not provided with alternative sources of thymine nucleotides, thymidylate synthase is an essential enzyme required for survival. Thymidylate synthase has therefore been identified as a major target for cancer chemotherapy (Danenberg, 1977). Jones *et al.* (1981) described the properties of a potent thymidylate synthase inhibitor, N^{10}-propargyl-5,8-dideazofolic acid (CB3717), which is a tight-binding quinazoline derivative competitive with 5,10-ch_2FH_4. It has been shown to be an efficient inhibitor of thymidylate synthase both *in vivo* and *in vitro* and to have significant anti-tumor activity. In the course of testing CB3717, Jackman *et al.* (1986a) were able to derive a cell line that was resistant to its cytotoxic effects. This cell line, L1210:C15, was derived from the murine cell line L1210 and had 45-fold elevated thymidylate synthase enzymatic activity. Kinetic parameters indicated that the overproduced enzyme was not different from that produced by wild type L1210 cells, and transport of the drug within L1210:C15 cells was also unaltered.

The messenger RNA for mouse thymidylate synthase has been cloned by Geyer and Johnson (1984) and that of human thymidylate synthase by Ayusawa *et al.* (1984). The isolation of the mouse cDNA clone pMTS-3, reported by Geyer and Johnson (1984), has enabled us to study directly the molecular basis of thymidylate synthase overproduction in L1210:C15.

In this paper we report that the TS[1] gene is amplified in L1210:C15 and show that this correlates with elevated levels of thymidylate synthase mRNA and enzymatic activity. Jenh *et al.* (1985a) have reported similar data on a thymidine synthase overproducer cell line LU3-7, that is resistant to fluorodeoxyuridine. L1210:C15 differs from this cell line with respect to the stability of the amplified TS gene and our studies extend those of Jenh *et al.* (1985a). We also show that the DHFR gene is amplified and overexpressed in L1210:C15. Finally, we demonstrate that the level of RNA for thymidylate synthase is invariant during the cell cycle and that amplification of the TS gene does not alter its temporal mode of expression.

MATERIALS AND METHODS

Cell Lines and Tissue Culture Media—L1210, a mouse lymphoblastoid cell line, was grown in RPMI 1640 medium supplemented with 20 mM Hepes, 2 mM glutamine, and 10% donor horse serum (Flow Laboratories, Great Britain). L1210:C15, a clonal cell line derived by serial selection of L1210 in increasing concentrations of CB3717 (Jackman *et al.*, 1986a), was grown as L1210 but the medium further supplemented with 500 μm of CB3717. L1210:C15$^-$ is L1210:C15 grown in the absence of selection for 375 generations. The levels of thymidylate synthase and dihydrofolate reductase enzymatic activity, compared to L1210 cells, for L1210:C15 are about 45- and 3-fold elevated and for L1210:C15$^-$ are about 40- and 4-fold elevated, respectively.

Mouse LmTK$^-$, APRT$^-$ cell line is a clonal derivative of the L cell line and was a gift of C. J. Marshall (Institute of Cancer Research). It lacks thymidine kinase and adenine phosphoribosyltransferase activities.

Elutriation of L1210 Cells—L1210 cells for elutriation were grown in RPMI 1640 medium supplemented with 20 mM Hepes, 10% donor horse serum, and 6 mM glutamine to a density 5×10^5 cells/ml and labeled with 10 μM 5-bromodeoxyuridine for 15 min. A total of 2×10^9 cells were harvested and resuspended in 100 ml of ice-cold phosphate-buffered saline. Cells were size-fractionated in ice-cold phosphate-buffered saline using a Beckman JE-10x elutriation rotor and 9 or 10 fractions of approximately equal cell numbers were collected. About 10^6 cells from each fraction were analyzed by flow cytometry using DNA content, measured by propidium iodide fluorescence, and cell size to determine their position along the cell cycle. Complementary analysis was also performed using an anti-5-bromodeoxyuridine monoclonal antibody to detect cells that had incorporated the 5-bromodeoxyuridine label, representing cells in S-phase of the cell cycle (Gratzner, 1982).

Preparation of Total Cellular RNA—Total cellular RNAs were prepared by modifications of the procedure of Auffray and Rougeon (1980). Frozen or fresh cell pellets (typically 5×10^7–10^8) were sonicated using MSE Soniprep 150 in 5 ml of 6 M urea, 3 M LiCl, and

* This work was supported by the Medical Research Council and Cancer Research Campaign. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: TS, thymidylate synthase; DHFR, dihydrofolate reductase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; kb, kilobase pairs; APRT, adenine phosphoribosyltransferase.

RNA precipitated overnight at 4 °C. Pelleted RNA was washed in 10 ml of the same solution, resuspended in 5 ml of 10 mM Tris, pH 7.2, 0.5% SDS, 50 μg/ml proteinase K, and incubated at 37 °C following which samples were extracted with phenol and chloroform and precipitated with 0.6 volumes of propan-2-ol in the presence of 0.3 M sodium acetate. Pellets were resuspended in sterile distilled water and stored at −20 °C in the presence of 2 units/ml of human placental ribonuclease inhibitor. RNA integrity was confirmed by electrophoresis in 1% agarose gels, containing 0.1% SDS plus E buffer (40 mM Tris, pH 7.6, 20 mM sodium acetate, 2 mM EDTA), and RNA visualized by staining in 0.5 mg/ml ethidium bromide.

Analysis of mRNAs—Northern blot analyses were performed essentially as described by Thomas (1980). RNA was denatured by a modification of the procedure of Lehrach *et al.* (1977) in 45% formamide, 14% formaldehyde, and 80 mM sodium phosphate, pH 6.8, at 70 °C for 5 min electrophoresed through 1% agarose gels containing 17% formaldehyde and 20 mM sodium phosphate, pH 6.8, for 16 h at 40 V. Running buffer (20 mM sodium phosphate, pH 6.8) was circulated throughout the duration of electrophoresis. RNA was transferred to Hybond-N membranes (Amersham Corp.) in 20 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and fixed according to the manufacturers' instructions. Membranes were prehybridized in a solution containing 5 × SSC, 5 × Denhardt's is 0.02% Ficoll, 0.02% bovine serum albumin (Pentex fraction V), and 0.02% polyvinyl pyrolidone), 50 mM sodium phosphate, pH 6.8, 0.1% SDS, 250 μg/ml sonicated and denatured salmon sperm DNA, 10 μg/ml poly(A), 10 μg/ml poly(C), and 50% formamide. Membranes were hybridized at 42 °C for 16 h in the solution described above and containing 10^6 cpm/ml of ^{32}P-labeled probe DNA. Some hybridizations also contained 10% (w/v) dextran sulfate. Probes were made by the method of Feinberg and Vogelstein (1983) using 450 Ci/mmol of [α-^{32}P]dCTP (Amersham International, Amersham, Great Britain) as the sole radiolabeled nucleotide. DNA was routinely labeled to a specific activity of 2–3 × 10^8 cpm/μg. Post-hybridization washes were in 2 × SSC, 0.1% SDS for 5 min at room temperature, followed by 2 × SSC, 0.1% SDS for 30 min at 65 °C and finally 0.1 × SSC, 0.1% SDS for 30 min at 65 °C. Membranes were autoradiographed at −70 °C using Kodak X-AR5 film and Du Pont QIII intensifying screens. The thymidylate synthase probe used was a 750-base pair *PstI* fragment of the mouse thymidylate synthase cDNA purified by agarose gel electrophoresis from the recombinant pMTS-3 (Geyer and Johnson, 1984). The probe used for dihydrofolate reductase analysis was a gel purified 735-base pair *Hin*dIII-*Bgl*II fragment of the mouse dihydrofolate reductase cDNA from pSV2-dihydrofolate reductase (Subramani *et al.*, 1981). Actin RNA was detected with a 2.3-kb *Pst* fragment sub-cloned from a mouse genomic actin clone,[2] and histone RNA with a 3.3-kb *Eco*RI chicken genomic clone containing the H_2A, H_2B, and H_1 sequences (D'Andrea *et al.*, 1981). Relative mRNA levels were estimated by microdensitometry of autoradiographs using a Joyce-Loebel Chromoscan-3 microdensitometer. A raster scan was carried out on a small, generally 10 × 10 mm, square containing the image of the radioactive mRNA. The total absorbance of this area is then used as a measure of mRNA levels. Care was taken to avoid reciprocity failure or saturation by using appropriate autoradiographic exposures.

DNA Analysis—Fresh or frozen cell pellets were dispersed in 5–10 ml of 0.1 M Tris-Cl, pH 7.5, 0.1 M NaCl, 10 mM EDTA, and SDS added to 1% w/v. Proteinase K was added to 50 μg/ml followed by incubation at 55 °C for 60 min. The DNA was then purified by routine procedures (Maniatis *et al.*, 1982) and dissolved in TE buffer (0.1 M Tris-HCl, pH 7.5, 1 mM EDTA). Restriction enzyme digests were performed according to the manufacturers' instructions and between 10–20 μg of digested DNAs per track were electrophoresed through 1% agarose gels and transferred to Hybond-N membranes in 10 × SSC according to the manufacturers' instructions. Prehybridization, hybridization, and post-hybridization washes and autoradiography were as for RNA analyses.

Probe Removal from Hybond-N Membranes—Probe removal from Hybond-N membranes for re-use of Southern and Northern blots was carried out by washing membranes in 50% formamide, 10 mM sodium phosphate, pH 6.8, at 65 °C for 1 h.

DNA-mediated Gene Transfers—Mouse LmTK$^-$, APRT$^-$ cells for DNA transfection experiments were plated at a density of 2 × 10^6 cells/150-mm plates, and gene transfers were performed by co-precipitating high molecular weight DNA with calcium phosphate, as described by Wigler *et al.* (1979). The precipitate was applied to cells for 18 h in Dulbecco's modified Eagle's medium plus 10% calf serum. Cells were then washed in 25 mM Tris, 140 mM NaCl, 5 mM KCl, 7 mM Na_2HPO_4, 0.1% (w/v) dextrose, 20 mM Hepes, pH 7.1, 5% (v/v) calf serum to remove the excess precipitate and fresh Dulbecco's Modified Eagle's medium plus 10% calf serum added. Cells were grown in the absence of selection for 20 h. Selection for APRT$^+$ phenotype was by the addition of 4.5 μg/ml azaserine and 13.5 μg/ml adenine to the growth medium and was maintained until APRT$^+$ cell colonies became visible (about 14 days). Plates were washed in phosphate-buffered saline, fixed in 4% formaldehyde, 85 mM NaCl, 100 mM Na_2SO_4, and stained with a 1% solution of crystal violet. Selection for transfer of TS gene from the thymidylate synthase overproducing cell line L1210:C15 was by adding 50 μM CB3717 to the growth medium and maintaining this selection for at least 2 weeks. Assay plates were then washed and stained as above.

[2] K. R. Willison, unpublished observations.

RESULTS

We wished to investigate several questions in relation to the higher level of TS in L1210:C15 compared to L1210. Is increased thymidylate synthase activity due to amplification of the TS gene and does amplification of the TS gene result in a corresponding elevation of thymidylate synthase mRNA levels? Is the DHFR gene amplified and are thymidylate synthase mRNA levels grossly different in L1210 and L1210:C15? Finally, we wished to investigate the stability of the overproduction of thymidylate synthase in the absence of continuous selection with CB3717.

Amplification of the Thymidylate Synthase Gene—Preliminary experiments were carried out to investigate the possibility that a single TS gene could be responsible for the overproduction of the enzyme. We reasoned that if the elevation of thymidylate synthase activity was due to a mutation in a single gene then it should be possible to transfer this to recipient cells and confer resistance to the cytotoxic effects of CB3717. High molecular weight DNA from the overproducer cell line L1210:C15 was used for gene transfer experiments employing the mouse LmTK$^-$, APRT$^-$ cell line as the recipient, with 50 μM CB3717 as the selection agent for TS gene transfer. Transfer of the APRT gene to the recipient cell line was used as a control to determine the efficiency of DNA transfections. Under conditions which resulted in efficient transfer of the APRT gene, there was no transfer of resistance to CB3717. We interpreted this result to mean that a single TS gene was not responsible for the overproduction of thymidylate synthase enzyme and consequent resistance to CB3717.

The possibility of TS gene amplification was directly investigated by Southern blot hybridizations of L1210 and L1210:C15 DNAs. We used a DNA dilution technique to assess the copy number of the TS gene in L1210:C15 (Fig. 1*A*). An equal mass of *Eco*RI-digested DNA from the two cell lines was loaded and also systematically decreasing quantities of L1210:C15. Amplification can be identified by inspection of the intensity of hybridization signal of the samples of equal mass and the extent of amplification by identification of the greatest dilution of L1210:C15 DNA that gives a signal equal to that of the L1210. The data in Fig. 1*A* indicate a 50-fold amplification of the TS gene in L1210:C15.

We also digested L1210:C15 with *Hin*dIII and *Eco*RI and compared thymidylate synthase fragment patterns generated by these enzymes with those seen in L1210 (Fig. 1*B*). The majority of DNA fragments generated by *Hin*dIII and *Eco*RI are the same size in L1210 and L1210:C15, and this implies that there are no large rearrangements of gene structure in the amplified DNA. However, in Fig. 1*B*, we do see a less intensely hybridizing band of 2.5 kb, and this may represent a minor rearrangement. Analysis of thymidylate synthase mRNA levels by Northern blot hybridizations (Fig. 2; see also

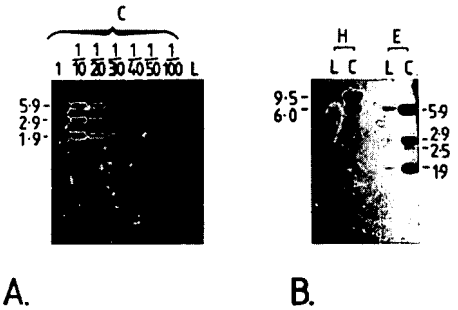

FIG. 1. **Amplification of the thymidylate synthase gene in L1210:C15.** *A*, dilution analysis by Southern hybridization of L1210 and L1210:C15 DNAs. *Tracks 1* and *L* contain 15 μg of *Eco*RI-digested L1210:C15 (*C*) and L1210 (*L*) DNA, respectively. Tracks labeled 1/10, 1/20, 1/30, 1/40, 1/50, and 1/100 contain the appropriately reduced amount of L1210:C15 DNA, *i.e.* 1/10 contains 1.5 μg of DNA. After hybridization with pMTS-3, the signal generated by 0.3 μg of L1210:C15 DNA (1/50) is the same as that generated by 15 μg of L1210 DNA implying that the TS genes are 50-fold amplified in L1210:C15. *B*, *Hin*dIII (*H*) and *Eco*RI (*E*) digests of 15 μg of L1210 (*L*) and L1210:C15 (*C*) DNA, hybridized with the thymidylate synthase probe. Note the faint 2.5-kb fragment in the *Eco*RI digest of L1210:C15 that is not visible in the L1210 track. Sizes are in given kb.

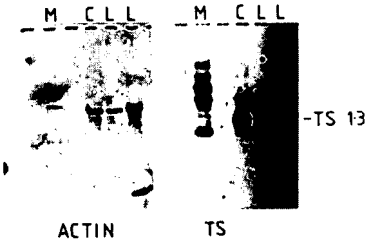

FIG. 2. **Thymidylate synthase mRNA levels in L1210 and L1210:C15.** Northern hybridization of about 30 μg of total RNA from L1210:C15 (*C*) or 30 or 60 μg of L1210 RNA (*L*). The *panel* labeled *TS* is hybridized with pMTS-3 and a strongly hybridizing thymidylate synthase mRNA of 1–3 kb is clearly visible in *track C*. The exposure of thymidylate synthase mRNA in L1210 (*L*) is very low in this picture (see also Figs. 4*B* and 5 for a similar experiment). The filters were then stripped of the thymidylate synthase probe and rehybridized with the actin probe (*panel* labeled *actin*) to control for loading and integrity of mRNAs. *M* is a marker track of single-stranded *Hin*dIII cleaved pM2 DNA. Thymidylate synthase mRNA is 1.3 kb.

Figs. 4*B* and 5) showed approximately 50-fold higher thymidylate synthase mRNA levels in L1210:C15 compared to the parental L1210 cell line, and the result was confirmed by dilution experiments (not presented). The elevation of thymidylate synthase mRNA is consistent with the 45-fold elevation of levels of thymidylate synthase enzymatic activity in L1210:C15 (Jackman *et al.*, 1986a).

Dihydrofolate Reductase Amplification—Jackman *et al.* (1986a) reported that dihydrofolate reductase activity was 2.6- to 3.0-fold elevated in L1210:C15. Hybridization of the membranes previously used to analyze thymidylate synthase DNA and mRNA (data presented in Figs. 1*A* and 2) with a probe for dihydrofolate reductase shows that the DHFR gene has also amplified, with an estimated copy number of 30–40 genes compared to the parental cell line (Fig. 3*A*). There is a 7-fold increase in dihydrofolate reductase mRNA levels (Fig. 3). There is a small disproportion between dihydrofolate reductase enzymatic activity (2.6 to 3.0-fold compared to L1210) and mRNA levels (about 7-fold increased) and gene copy number (30-fold increase). We see no significant levels of rearrangements of dihydrofolate reductase DNA fragments in L1210:C15 compared to L1210.

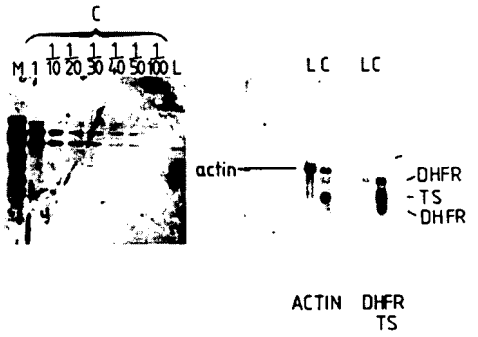

FIG. 3. **Analysis of dihydrofolate reductase genes and mRNA.** *A*, dilution analysis of DHFR gene copy marker. The filters used for the experiment detailed in Fig. 1 were stripped of the thymidylate synthase probe and rehybridized with the dihydrofolate reductase probe. Identical logic to that used in Fig. 1 indicated the DHFR gene is amplified 30- to 40-fold. *M* is a marker track of radiolabeled phage λDNA cut with *Hin*dIII mixed with DNA cut with *Hin*dIII plus *Eco*RI; *L* is L1210 DNA; *C* is L1210:C15 DNA. *B*, dihydrofolate reductase mRNA levels. Samples of total mRNA from L1210 (*L*) and L1210:C15 (*C*) were hybridized with the dihydrofolate reductase and thymidylate synthase probes together (*DHFR* and *TS panel*). The dihydrofolate reductase probe detects two mRNAs. The filters were then stripped and rehybridized with the actin probe (*panel* labeled *Actin*). Microdensitometry of the autoradiograph and normalization of RNA levels with the actin mRNA leads to the conclusion that dihydrofolate reductase is 7-fold elevated in L1210:C15 compared to L1210.

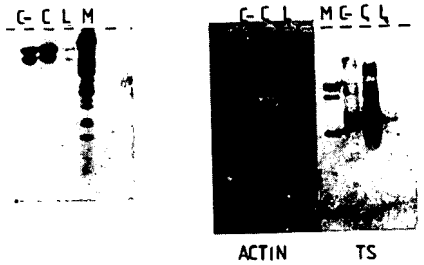

FIG. 4. **Stability of the amplified TS genes in the absence of selection.** *A*, *Eco*RI-digested DNA from L1210 (*L*), L1210:C15 (*C*), and L1210:C15- (C^-) hybridized with the thymidylate synthase probe. Although levels of TS gene fragments in C and C^- appear similar, there is twice as much DNA in C^- as C, leading to the conclusion that L1210:$C15^-$ has 2- to 3-fold less TS genes than L1210:C15. *M* is a marker track as described in the legend to Fig. 3. *B*, thymidylate synthase mRNA levels. Equal amounts of total RNA from L1210 (*L*), L1210:C15 (*C*), and L1210:$C15^-$ were analyzed by Northern hybridization. The *panel* labeled *actin* is the control for equal loading and mRNA integrity, and the *panel* labeled *TS* is the analysis of thymidylate synthase mRNA in the same cell lines. We note a reduction of about 3- to 5-fold of thymidylate synthase mRNA level in L1210:$C15^-$ compared to L1210:C15. *Track* labeled *M* is a marker as described in the legend to Fig. 2.

Stability of the Amplified DNA—Amplified DNA in eukaryotic cells is often unstable in the absence of selection (Stark and Wahl, 1984). The stability of the amplified thymidylate synthase locus was investigated by Southern analysis of DNA from a cell line, L1210:$C15^-$, that had been grown in the absence of CB3717 selection for 300 generations. Fig. 4*A* shows that there is only a 2- to 3-fold reduction of TS gene gene copy number in the prolonged absence of drug selection, in marked contrast to the similar studies of other TS gene amplified cell lines (Jenh *et al.*, 1985a), which showed rapid loss of amplified gene copies with a half-life of about 30 days.

Analysis of mRNA from L1210:C15 (Fig. 4*B*) showed a 3- to 5-fold decrease in thymidylate synthase mRNA in L1210:C15$^-$ compared to L1210:C15, which is compatible with the reduced gene copy number in L1210:C15$^-$.

Control of Thymidylate Synthase mRNA Levels—Jenh *et al.* (1985b) showed that there were significantly higher levels of thymidylate synthase mRNA in exponentially growing compared to quiescent cells. We wish to see if similar observations could be made on L1210:C15 and L1210:C15$^-$ or whether the elevated thymidylate synthase mRNA levels in these cells resulted in different controls of gross thymidylate synthase mRNA levels.

In Fig. 5, we show the results of Northern analysis of RNA isolated from L1210, L1210:C15, and L1210:C15$^-$. *Tracks* labeled *H* contain RNA samples from the quiescent cells grown to high density (1.5×10^6 cells/ml). *Tracks* labeled *Lo* contain RNA made from cells growing exponentially and at a density of 1.5×10^5/ml. There is an accumulation of thymidylate synthase mRNA in cycling compared to quiescent cells, and we can see no significant difference in the ratio of cycling to quiescent thymidylate synthase mRNA in L1210:C15 and L1210:C15$^-$.

Accumulation of particular mRNA species in cycling compared to quiescent cells has been reported for several gene transcripts. However, such accumulation is rarely accompanied by differential distribution of mRNA within the different phases of the undisturbed cell cycle (Thompson *et al.*, 1985). We wished to investigate the distribution of thymidylate synthase mRNA within the normal cycle of both L1210 and L1210:C15 cells separated into different phases of the cycle by elutriation (described under "Materials and Methods"). Flow cytometric methods were used to assign actual position within the cycle, and we were able to divide cells into 9 or 10 populations using these techniques.

Northern blots of RNA from elutriated cells show that actin mRNA levels do not vary within the cycle (Thompson *et al.*, 1985) and histone mRNAs accumulate in S-phase (Osley and Hereford, 1982; Thompson *et al.*, 1985). Control hybridizations of the L1210 RNA were carried out using probes ("Materials and Methods") specific for both these types of mRNAs (Fig. 6) and microdensitometry of the actin mRNA levels was used to normalize loading of total RNA (Table I). Histone mRNAs accumulate as cells enter S-phase, as expected. We

FIG. 5. **Effect of growth state of cells on thymidylate synthase mRNA levels.** RNA was made from cells at high density (*H*) or low density (*Lo*) as described in the text, and hybridized with the thymidylate synthase probe (*panel* labeled *TS*). *Tracks* labeled *L* contain mRNA from L1210, *tracks* labeled *C*$^-$ are from L1210:C15$^-$, and *tracks* labeled *C* are from L1210:C15. The *panel* labeled *Actin* is the actin probe rehybridization of the same filter area for the thymidylate synthase probe. Normalization of the various samples for loading using the actin mRNA leads us to conclude that thymidylate synthase mRNA levels are reduced in cells grown at high density and elevated in cells, growing exponentially, at low density. We see no difference in induction ratios between all three cell types. *M* are marker tracks as described in the legend to Fig. 2.

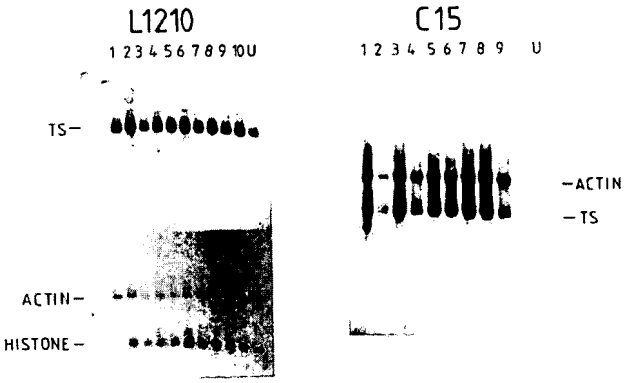

FIG. 6. **Cell cycle control of thymidylate synthase mRNA.** Total RNA isolated from elutriation fractions of L1210 or L1210:C15 (labeled *C15*) was analyzed for thymidylate synthase, actin, or histone mRNAs. *1–10* are L1210 elutriation fractions and *1–9* are similar fractions of L1210:C15 cells. *U* are samples of unfractionated cell RNA. For L1210, actin and histone probes were used simultaneously, followed by the thymidylate synthase probe. For L1210:C15, actin and thymidylate synthase were hybridized simultaneously. The various mRNA levels were estimated by microdensitometry of the autoradiograph, and actin mRNA levels were used to normalize for variation in loading. These data are tabulated in Table I.

TABLE I

Summary of elutriation experiments carried out on L1210 or L1210:C15 cells

The proportion of cells in S-phase as judged by 5-bromodeoxyuridine incorporation and flow cytometry is given for each fraction. Loading of total RNAs were normalized relative to actin mRNA. After normalization, levels of histone and thymidylate synthase mRNAs are expressed, arbitrarily, relative to the amount of each mRNA in fraction 1 of the L1210 cells and thymidylate synthase mRNA levels similarly analyzed from L1210:C15. mRNA levels were estimated by microdensitometry of autoradiographs as detailed under "Materials and Methods."

Fraction No.	L1210 cells		Cells in S-phase	L1210:C15	
	Histone	Thymidylate synthase		Thymidylate synthase	Cells in S-phase
		%		%	
1	1.00	1.00	5	1.00	26
2	1.66	1.30	7	0.86	40
3	3.15	1.36	29	0.77	52
4	3.57	1.73	28	0.56	57
5	2.50	1.60	55	0.65	67
6	3.77	1.43	72	0.64	73
7	4.53	1.45	84	0.68	71
8	3.81	1.55	81	0.72	76
9	3.24	1.31	87	0.50	83
10	2.49	1.00			

concluded from these control experiments that successful separation of the cells by cycle position had been achieved. The filters were then rehybridized with the thymidylate synthase probe (Fig. 6). L1210:C15 RNA samples were hybridized with actin and thymidylate synthase probes simultaneously. Careful microdensitometry of the autoradiograph of the thymidine synthase mRNA hybridization and normalization for RNA loading against actin mRNA levels leads us to conclude that there is no significant variation in thymidylate synthase mRNA levels throughout the cycle in either L1210 or L1210:C15 cells.

DISCUSSION

In this paper we report the amplification of the gene for thymidylate synthase in the murine cell line L1210:C15 which was derived by selective growth on increasing concentrations

of the thymidylate synthase competitive inhibitor CB3717. Direct analysis of the TS gene by Southern blotting showed that the gene for thymidylate synthase has amplified 40- to 50-fold in the resistant cell line compared to the parental line, and Northern analysis showed that there was a concomitant 40- to 50-fold elevation of thymidylate synthase mRNA levels. These data are compatible with enzyme activity assays which show a 45-fold elevation in L1210:C15 compared to the parental cell line (Jackman *et al.*, 1986a).

Comparison of the restriction fragment sizes of the amplified TS gene and the normal allele shows that most of the amplification has occurred without rearrangement of hybridizing fragments. There is, however, a novel band in the *Eco*RI digest of L1210:C15 genomic DNA at 2.5 kb, and this may represent either a minor rearrangement of the gene present in a subset of amplified sequences or a genomic fragment present in the parental cell line DNA that has only a small region of homology with the probe and is thus detectable only when amplified. Aberrant fragments in amplified DNA have been reported by other workers (Debatisse *et al.*, 1986). We also confirm the finding of Jenh *et al.* (1985a), who noted significant polymorphisms of fragment size in different mouse cell lines.

Analysis of L1210:C15$^-$, grown in the absence of CB3717 selection for 375 generations, showed only a 2- to 3-fold reduction of TS gene copy number in L1210:C15$^-$ in 12 months compared to a 5- to 10-fold reduction in 3 months for LU3-7 (Jehn *et al.*, 1985a), suggesting that TS gene amplification in the cell line C15:L1210 is more stable than LU3-7. Instability of amplified DNA in the absence of selection has often been associated with the presence of double minute chromosomes within the cell (Cowell, 1982). Cytological analysis of L1210 and L1210:C15[3] failed to detect such chromosomes. Amplified genes have also been shown to be contained within a homogeneously staining region of a chromosome (Stark and Wahl, 1984). L1210 and L1210:C15 are aneuploid, and it is difficult to identify homogeneously staining regions for this reason. We would anticipate that amplified TS genes in L1210:C15 are contained within a "normal" L1210 chromosome, and under these conditions are relatively stable in the absence of selection.

Our Northern analysis of elutriated L1210 and L1210:C15 cell line RNAs demonstrates unequivocally that thymidylate synthase RNA levels show no detectable variation within the cell cycle. This is in agreement with the data of Rode *et al.* (1980) showing thymidylate synthase enzyme activity in crude extracts of cells synchronized by chemical block to be constant in different phases of the L1210 cell cycle. However, thymidylate synthase enzyme activity measured in whole cells from the same synchronization experiment showed the activity reaching a maximum in early S-phase, indicating post-translational control. Studies of Jehn *et al.* (1985b) show an elevation of thymidylate synthase mRNA as cells enter S-phase after quiescent cells were serum-stimulated. We would emphasize that observation of mRNA accumulation occurring during the transition of cells from quiescence to proliferation need not imply similar accumulation during the same phase of the normal cell cycle. Thompson *et al.* (1985) showed that, although c-*myc* steady-state mRNA level undergoes a transient increase within 2 h of serum stimulation of quiescent cells, the level of c-*myc* mRNA was constant in the cell cycle of actively dividing cells separated into different phases of the cycle by elutriation. Similar results were obtained for β-actin. Our Northern analysis of elutriated cell RNA demonstrates invariant levels of thymidylate synthase mRNA in the unperturbed cell cycle. Amplification of the TS gene does not in any way alter its temporal expression as thymidylate synthase mRNA levels in the cell cycle of L1210:C15 cells are similarly invariant.

Jackman *et al.* (1986a) demonstrated a 2.6 × increase in dihydrofolate reductase enzyme activity in L1210:C15, but our Southern blot analysis of L1210:C15 DNA shows that the gene for dihydrofolate reductase is amplified about 30- to 40-fold with a corresponding increase in RNA levels (Fig. 3, *A* and *B*). There is a discrepancy between gene copy number and mRNA levels, and this has been seen in the case of other amplified genes (Debatisse *et al.*, 1986).

The finding that the DHFR gene is amplified in L1210:C15 was itself unexpected since the properties of CB3717 would suggest its target is thymidylate synthase. We can exclude physical linkage of the mouse TS and DHFR gene as an explanation of co-amplification, which has been reported in other cases (Wahl *et al.*, 1983). Jenh *et al.* (1985a) report that the cell line, M50L3, contains amplified DHFR but not TS genes; TS gene amplification was subsequently induced leading to the isolation of the cell line, LU3-7, that was resistant to the cytotoxic effects of fluorodeoxyuridine. There was no apparent change in DHFR gene copy number during this experiment, implying that the DHFR and TS genes are not closely linked. Another possible explanation for co-amplification is that CB3717 has been shown to be contaminated by low (less than 1%) levels of 5,8-dideazafolic acid (CB3705) (Jackman *et al.*, 1986b), and the contamination has significantly greater affinity for dihydrofolate reductase than thymidylate synthase. It is likely that at the highest concentrations of CB3717 (500 μM) that were used to derive L1210:C15, the concentration of CB3717 and CB3705 are sufficient to inhibit dihydrofolate reductase activity. Selection of a rapidly growing cell line would then require elevated dihydrofolate reductase and thymidylate synthase enzymatic activity, and we would predict that amplification of DHFR genes would occur late in the selection process based upon this model.

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[3] A. M. A. Imam, B. Reeves, and P. F. R. Little, unpublished observations.

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Correlation of amplification and overexpression of the c-*myc* oncogene in high-grade breast cancer: FISH, *in situ* hybridisation and immunohistochemical analyses

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In this study, we analysed gene amplification, RNA expression and protein expression of the c-*myc* gene on archival tissue specimens of high-grade human breast cancer, using fluorescent *in situ* hybridisation (FISH), nonradioactive *in situ* hybridisation and immunohistochemistry. The specific question that we addressed was whether expression of c-Myc mRNA and protein were correlated with its gene copy amplification, as determined by FISH. Although c-Myc is one of the most commonly amplified oncogenes in human breast cancer, few studies have utilised *in situ* approaches to directly analyse the gene copy amplification, RNA transcription and protein expression on human breast tumour tissue sections. We now report that by using the sensitive FISH technique, a high proportion (70%) of high-grade breast carcinoma were amplified for the c-*myc* gene, irrespective of status of the oestrogen receptor. However, the level of amplification was low, ranging between one and four copies of gene gains, and the majority (84%) of the cases with this gene amplification gained only one to two copies. Approximately 92% of the cases were positive for c-*myc* RNA transcription, and essentially all demonstrated c-myc protein expression. In fact, a wide range of expression levels were detected. Statistically significant correlations were identified among the gene amplification indices, the RNA expression scores and protein expression scores. c-*myc* gene amplification, as detected by FISH, was significantly associated with expression of its mRNA, as measured by the intensity of *in situ* hybridisation in invasive cells ($P = 0.0067$), and by the percentage of invasive cells positive for mRNA expression ($P = 0.0006$). c-*myc* gene amplification was also correlated with the percentage of tumour cells which expressed high levels of its protein, as detected by immunohistochemistry in invasive cells ($P = 0.0016$). Thus, although multiple mechanisms are known to regulate normal and aberrent expression of c-*myc*, in this study, where *in situ* methodologies were used to evaluate high-grade human breast cancers, gene amplification of c-*myc* appears to play a key role in regulating expression of its mRNA and protein.
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Keywords: c-*myc*; breast cancer; gene amplification; gene expression

The c-*myc* oncogene has been shown to be amplified and/or overexpressed in many types of human cancer (Marcu *et al*, 1992; Nass and Dickson, 1997; Nesbit *et al*, 1999; Liao and Dickson, 2000). Numerous experiments *in vivo* have also causally linked aberrant expression of this gene to the development and progression of cancer in different body sites (Marcu *et al*, 1992; Nass and Dickson, 1997; Nesbit *et al*, 1999; Liao and Dickson, 2000). However, several critical issues regarding the significance of c-*myc* in human cancer still remain obscure. First, even for a given type of malignancy, the frequencies of the alterations of c-*myc* at the cytogenetic and expression levels vary greatly from one report to another (Liao and Dickson, 2000). For instance, the frequencies of its amplification, mRNA and protein overexpression in breast cancer vary between 1–94, 22–95 and roughly 50–100%, respectively, among different reports (Liao and Dickson, 2000). Thus, it is still unclear to what extent this gene is altered at the cytogenetic level and at different expression levels in breast carcinoma.

One controversial issue pertains to the prognostic value of c-*myc* gene alterations in cancer. The central role of c-Myc protein in accelerating cell proliferation, documented by many early studies, has led to a general concept for many types of cancer that amplification or overexpression of this gene may be associated with a more aggressive tumour and a poorer patient survival (Berns *et al*, 1992; Marcu *et al*, 1992; Sato *et al*, 1995; Nass and Dickson, 1997; Nesbit *et al*, 1999; Visca *et al*, 1999; Liao and Dickson, 2000). However, many reports have shown an opposite correlation (Sikora *et al*, 1985, 1987; Watson *et al*, 1986; Polacarz *et al*, 1989; Voravud *et al*, 1989; Williams *et al*, 1990; Melhem *et al*, 1992; Pietilainen *et al*, 1995; Diebold *et al*, 1996; Smith and Goh, 1996; Augenlich *et al*, 1997; Bieche *et al*, 1999), while other studies do not support either of these conclusions. For instance, gene amplification or overexpression of c-Myc protein has also been shown to associate with a better tumour differentiation or a better

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patient survival for cancer of the testis, ovary, bile ducts, colon and breast (Sikora *et al*, 1985, 1987; Watson *et al*, 1986; Polacarz *et al*, 1989; Voravud *et al*, 1989; Williams *et al*, 1990; Melhem *et al*, 1992; Pietilainen *et al*, 1995; Diebold *et al*, 1996; Smith and Goh, 1996; Augenlich *et al*, 1997; Bieche *et al*, 1999). This controversy does not appear to be related completely to the cancer type, since both positive (Berns *et al*, 1992; Visca *et al*, 1999) and negative (Williams *et al*, 1990; Melhem *et al*, 1992; Pietilainen *et al*, 1995; Smith and Goh, 1996; Augenlich *et al*, 1997; Bieche *et al*, 1999) correlations have been reported for colon cancer and breast cancer. More interestingly, c-Myc overexpression has been shown to predict a poorer prognosis for cutaneous melanoma, but a favourable outcome for uveal melanoma (Grover *et al*, 1997; Chana *et al*, 1998a, b, 1999; Grover *et al*, 1999). These data indicate different roles of c-Myc, even in the same type of tumour, perhaps depending upon different tissue microenvironments.

Another controversial issue concerns the nuclear-cytoplasmic localisation of c-Myc. Studies of neoplasms of the colon, testis, ovary and liver have shown that predominantly nuclear localisation of c-Myc tends to occur in benign lesions, while cytoplasmic localisation tends to occur in more malignant tumours (Sikora *et al*, 1985; Sundaresan *et al*, 1987; Melhem *et al*, 1992; Sasano *et al*, 1992; Yuen *et al*, 2001). Whether these patterns of subcellular localisation of c-Myc tend to reflect the malignant status of breast cancer remains an enigma.

A recent study of the impact of DNA amplification on gene expression patterns in breast cancer used mRNA and DNA from 14 breast cancer cell lines. Analysis was conducted with a 13 000 cDNA clone array for gene expression measurement and a Comparative Genomic Hybridisation (CGH) microarray for gene copy number measurements. This study also included known breast cancer genes, such as c-*myc*, *HER/2-neu* and *aib1* (Hyman *et al*, 2002). Interestingly, 44% of the most highly amplified genes were also overexpressed at the mRNA level. Consistent with this pattern, c-*Myc* gene copy number and its expression levels showed a statistically significant ($\alpha = 0.020$) correlation in this microarray study of breast cancer cell lines. Another study, by Pollack and colleagues, used microarray analysis and BAC array CGH of RNA and DNA (respectively) extracted from intermediate grade human breast tissues, and tested for amplification and expression of c-Myc (among other genes). This study demonstrated that two out of 37 specimens were both amplified and overexpressed, while others were either amplified or overexpressed, but not both. The authors of this study suggested that contaminating stromal tissue may compress the fluorescence ratios leading to underestimates of gene amplification and overexpression (Pollack *et al*, 2002).

To more clearly address the importance of gene amplification and expression of c-Myc in human breast cancer, we used *in situ* methodologies, which can clearly distinguish stromal and carcinoma components. We studied the amplification and over expression of the c-*myc* gene with fluorescent *in situ* hybridisation (FISH), non-radioactive *in situ* hybridisation (ISH) and immunohistochemical (IHC) approaches on paraffin-embedded biopsy sections of untreated, high-grade breast cancer. It was observed that 70, 92 and 70% of the cancer cases exhibited c-*myc* gene amplification, its mRNA overexpression and its protein over expression, respectively. In most of the cases (84%) that showed gene amplification, the c-*myc* gene gained only one to two copies, which is consistent with c-*myc* FISH data from other studies. Unlike some oncogenes, such as N-*myc*, which typically demonstrates gene amplification copy numbers of greater then 10 in neuroblastoma, and *HER-2/neu* (Sartelet *et al*, 2002), whose copy numbers range up to 14–40 in breast carcinomas (Isola *et al*, 1999), gene copy numbers of c-*myc* are not as greatly increased. In the study noted earlier, using breast cancer cell line CGH array and cDNA microarray expression analysis, it was demonstrated that the most dramatically increased expression levels were associated with large gene copy number increases, although low-level gains and losses had a significant influence on gene expression dysregulation (Hyman *et al*, 2002). Only one study has been published (Pollack *et al*, 2002) that has begun to determine if these findings are directly relevant to actual human breast tumour tissues, since many of the genetic changes in tissue culture cell lines are more extreme than those displayed in primary tumour material. Furthermore, the relationships among gene amplification, mRNA expression and c-Myc protein expression were not explored in prior human breast cancer cell line and tumour tissue studies (Hyman *et al*, 2002; Pollack *et al*, 2002).

In our human breast tumour tissue study, a high correlation was found between c-*myc* FISH and ISH, for both percentage of staining ($P<0.0067$) and intensity positive cells ($P<0.0006$). In addition, c-*myc* gene copy amplification by FISH was correlated with c-Myc protein expression positive cells by IHC ($P<0.0016$). These results support the idea that c-Myc overexpression of both mRNA and protein is related to the copy number of the *c-myc* DNA amplification. We show in this study that amplification and overexpression of c-Myc occur with high frequency in high-grade human breast cancer tissues.

MATERIALS AND METHODS

Materials

Formalin-fixed, paraffin-embedded tissue blocks of breast carcinoma and normal breast tissue were obtained from the Histopathology and Tissue Shared Resource at the Lombardi Comprehensive Cancer Center (LCCC), at Georgetown University Medical Center. The criteria for tumour selection were the following: negative progesterone receptor status, metastases to auxiliary lymph nodes and high grade (Elston Score >7). The oestrogen receptor status of the tumours was known from archived pathology reports. The parameters were chosen from our prior meta-analysis (Deming *et al*, 2000), as indications of a high likelihood of c-*myc* gene amplification. Normal breast tissue specimens were from reduction mammoplasty. Serial sections (5 μm) for FISH, ISH and IHC were prepared by the LCCC Histopathology and Tissue Shared Resource.

FISH

A dual-label FISH technique was used (Jenkins *et al*, 1997). Slides were baked overnight at 60°C to assure adherence of the sample. Tissue sections were deparaffinised with two successive, 10 min xylene washes, and then dehydrated in a graded ethanol series of 70, 80 and 95% at room temperature. Samples were then digested with 4% pepsin (Sigma, St Louis, MO, USA) at 45°C for 10 min. DNA probes used were an alpha satellite probe to chromosome 8, labelled with biotin, and a *c-myc* probe, labelled with digoxigenin (Ventana, Tucson, AZ, USA). Codenaturation was performed at 90° for 10 min on a hot plate. Hybridisation was at 37°C for 12–16 h. Detection of signals was accomplished with an antiavidin antibody labelled with Texas Red, and an antidigoxigenin antibody conjugated to fluorescein (Ventana, Tucson, AZ, USA). Slides were postwashed in 2 × SSC at 72°C for 5 min and counterstained with DAPI to visualise cell nuclei. Results were viewed and quantified with a Zeiss Axiophot fluorescence microscope, equipped with appropriate filters and an Applied Imaging Cytovision system (Pittsburgh, PA, USA). In this approach, the *c-myc* unique sequence probe was visualised as a green signal and the control probe for the chromosome 8 centromere was red, thus easily being distinguished when scored.

One serial section from each tumour sample was stained with haematoxylin and eosin and first reviewed by a pathologist (BS), to help identify the tumour area of the section. This procedure ensured that the tumour cells, but not the normal cells, were

counted. Nuclei of up to 50 tumour cells were scored from each FISH-stained section, independently by two investigators. Hybridisation signals were averaged, and the amplification index was presented as the number of c-*myc* signals divided by the number of chromosome 8 centromere signals. A 1.8-fold increase was used as the criterion to judge the presence of c-*myc* gene amplification.

In situ hybridisation

In situ hybridisation (ISH) was carried out with a nonradioactive method, described previously (Liao *et al*, 2000a, b). One serial section from each specimen was hybridised overnight at 60°C with riboprobes, that were *in vitro* transcribed from the antisense or sense strand of an approximately 300 bp cDNA of human c-*myc* (ATCC, Manassas, VA, USA), labelled with digoxigenin-conjugated UTP. The sections were then incubated with an antibody against digoxigenin, followed by incubation with a second antibody conjugated to alkaline phosphatase. The signal was visualised by colour development with 5 bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. All reagents were purchased from Boehringer Mannheim, Indianapolis, IA. To control the signal specificity, two serial sections were mounted on the same slide for hybridisation with the antisense and sense probes, respectively. ISH was given an intensity and percentage scores, based on intensity of positive staining and number of cells staining, respectively. Intensity scores were assigned 0, 1, 2 and 3, and percentage scores were assigned as 1- 1–25, 2- 26–50, 3- 51–75 and 4- 76–100%.

Immunohistochemistry

Immunohistochemical staining (IHC) was performed using an avidin–biotin complex (ABC) method described previously (Liao *et al*, 1998). One serial section of each specimen was deparaffinised and blocked with 3% peroxide. Antigens were retrieved by heating slides in a microwave oven in 50 mM citrate buffer, pH 6.4, at boiling temperature, for 12 min. After blocking with 6% normal goat serum, the section was incubated with a mouse monoclonal antibody to human c-Myc (9E10, Sigma Chemical Company, St Louis, MO, USA) at 1:100 dilution for 2 h, followed by 1 h incubation with a second antibody conjugated with biotin (Vector Laboratories Inc., Burlingame, CA, USA). The section was then incubated with peroxidase-conjugated avidin (Dako, Corporation, Carpinteria, CA, USA) for 30 min, followed by colour development with diaminobenzidine and peroxide. All procedures were carried out at room temperature. To control the signal specificity, serial sections from 10 tumour samples were also stained using an alternate c-Myc antibody (C19 from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:60 dilution. This antibody resulted in focally positive staining in the tumour, but the staining intensity was weaker. To control the signal specificity, serial sections were made from five selected positive cases which were subjected to the same staining procedure, with a normal mouse IgG to replace the c-Myc antibody. This control staining did not give rise to a signal, demonstrating the specificity of the c-Myc antibody signal. IHC staining was given an intensity and percentage score based upon the intensity of positive staining and number of cells staining. Intensity scores were assigned 0, 1, 2 and 3 and percentage scores were assigned as 1- 1–25, 2- 26–50, 3- 51–75 and 4- 76–100%. Determinations were made of cellular localisation of c-Myc antibody staining to cytoplasm and/or nucleus in normal and invasive cells within each breast tumour specimen.

Statistical analyses

For each analysis of gene copy amplification (FISH), mRNA expression (ISH) and protein expression (IHC), all cases were first grouped as positive or negative to calculate the percentages of positive cases and negative cases, as described (Zar, 1974). Fisher's exact test was used to compare percentages, and two-sample *t*-test or Wilcoxon rank test was used to compare average scores. Both ISH and IHC were given intensity and percentage scores, based on intensity of positive staining and number of cells staining, respectively. As noted earlier, intensity scores were assigned 0, 1, 2 and 3 and percentage scores were assigned as 1- 1–25, 2- 26–50, 3- 51–75 and 4- 76–100%. A score of >2 for either intensity of staining or percentage of cells positive by ISH was assigned as high. For IHC, an intensity score of >1 was assigned as high and a percentage score of >3 was categorised as high. Each amplification index was paired with its corresponding mRNA expression score to calculate the coefficient *r*. The same method was used to estimate the association of the amplification indices with the c-Myc protein expression levels, and the association of the mRNA expression levels with the protein expression levels. A *P*-value of 0.05 or less was used to determine the statistical significance in all analyses. In all, 54 pairs of normal *vs* invasive tissues were analysed using McNemars χ^2 test to determine if there was a difference in cellular localisation of c-Myc antibody signal to nuclear or cytoplasmic compartments.

RESULTS

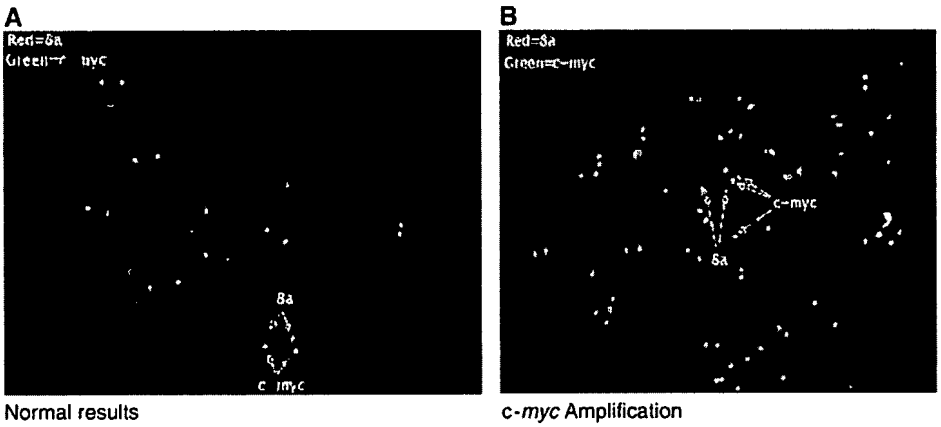
FISH analysis of gene amplification

Amplification of the c-*myc* gene was measured by a FISH test in 46 cases of breast cancer; Figure 1 demonstrates cells with no amplification (one copy of c-*myc* /one copy of chromosome 8 centromere, and a moderate amplification a 3/1 ratio). Amplification was calculated by the number of c-*myc* signals divided by the number of chromosome 8 alpha satellite signals. A 1.8-fold increase cut-off was used to judge gene amplification. As shown in Table 1, 32 out of 46 (70%) cases were gene amplified for c-*myc*, whereas only 30% (14/46) of the cases showed amplification indices lower than the cut-off value. The amplification indices for most (84%, or 27/32) cases with gene amplification, ranged between 1.8- and three-fold, indicating that the locus gained up to two copies of c-*myc* in the majority of the cases. The percentage of cases with gene gains of three copies or higher was 11% (five out of 46) of total cases analysed, or near 16% (five out of 32) of the cases with gene amplification, including one case (2% of total cases or 3% of the cases with gene amplification) with the highest index of 5 (a gain of four copies).

In all, 28 of the breast carcinomas in this study were ER negative, and 14 were ER positive. The average c-*myc* gene amplification score was 1.896 (s.e. = 0.196) for ER positive and 2.201 (s.e. = 0.157) for ER negative. Although ER-negative tumours had a slightly higher average c-*myc* score, the difference was not statistically significant (two-sided $P = 0.252$ from two-sample *t*-test and 0.251 from Wilcoxon rank test), consistent with the results of our prior meta-analysis of the literature (Deming *et al*, 2000).

In situ hybridisation analysis of c-*myc* mRNA expression

A total of 51 breast cancer samples were studied for c-Myc mRNA expression, with non radioactive *in situ* hybridisation (ISH). ISH results were assigned intensity and percentage scores based upon signal intensity of positive staining and number of cells staining within the sample, respectively. As shown in Table 2, 86% (44 out of 51) tumours were scored as high in intensity, and 92% (47out of 51) had more than 51% positive cells, also considered as highly increased c-Myc expression. mRNA expression was heterogeneous in the breast tumour tissue, and no morphologic subtype was predominant in the high or low categories. One case showed no c-Myc ISH staining. In 79% (38/48) of cases, epithelia in normal mammary glands adjacent to the tumour also showed a high



Normal results

c-*myc* Amplification

Figure 1 FISH analysis of c-*myc* amplification in tumour cells from breast tumour tissue sections. FISH probe for human c-*myc* unique-sequence is seen as green, while the normal control signal, a centromeric probe signal for chromosome 8 is shown in red. The nuclei of tumour cells were visualised by DAPI counter-staining. (**A**) 1 : 1 copy ratio of c-*myc* to chromosome 8 (c-*myc*/8 centromere), indicating no amplification of c-*myc* in tumour cells. (**B**) 1 : 3 copy ratio of c-*myc* to chromosome 8 (c-*myc*/8 centromere), a moderate amplification of the c-*myc* gene.

Table 1 c-*myc* gene copy amplification analysis by FISH in poor prognosis human breast tumour samples

Amplification index (#c-myc signals/# control signals)+	Percentage of samples with FISH ratios in each category	
1.0–1.7	30%	14 out of 46
1.8–1.99	20%	Nine out of 46
2.0–2.9	39%	18 out of 46
>3.0	11%	Five out of 46

Analysis was conducted on 46 individual paraffin-embedded tissue samples with negative progesterone receptor status, positive lymph node involvement and high tumour grade. +Normal control ratio is 1.

intensity of staining. In three cases, no staining was seen in the normal terminal duct lobular units. Figure 2 shows representative fields of high, medium and low *c-myc* mRNA expression levels in invasive ductal carcinoma samples.

Association of FISH and ISH

c-Myc scores were dichotomised as binary variables (high or low), and a score of 2 or higher was categorised as high on ISH. A score higher than median was categorised as high from FISH studies. These dichotomised scores are depicted in Table 3. A Fisher's exact test was performed for comparing binary responses to see if there was any association between FISH and ISH. It was found that the FISH score was significantly associated with percentage of staining in the invasive cells ($P=0.0067$, two-sided McNemar's test) and also with the intensity score on ISH ($P=0.0006$, two-sided).

Immunohistochemical staining of c-Myc proteins

In total, 51 breast carcinomas, which were subjected to FISH analysis, and all of which also had been analysed for c-*myc* mRNA by *in situ* hybridisation, were also analysed for the expression of c-Myc protein, using immunohistochemical staining with the 9E10 antibody. IHC results were assigned an intensity and percentage score based on intensity of positive staining and number of cells staining, respectively. Intensity scores were assigned 0, 1, 2 and 3 and percentage scores were assigned as 0, 1- 0–25, 2- 26–50, 3- 51–75 and 4- 76–100. For IHC, an intensity score of >1 was assigned as high and a percentage score of >3 was categorised as high. Figure 2 shows examples of high, medium and low levels of c-myc antibody staining in invasive ductal carcinoma samples. In 34 cases, normal tissue was seen; 30 of these showed cytoplasmic staining and 22 had nuclear staining in terminal ductal lobular units. In all, 12 cases showed 1+, 14 cases 2+ and four cases 3+ cytoplasmic staining. *In situ* hybridisation revealed positive staining in 46 out of 49 cases with normal tissue. Seven cases showed 1+, 13 cases showed 2+ and 26 cases showed 3+ staining by ISH. Both immunohistochemistry and *in situ* hybridisation showed diffuse positivity in adipocytes.

Table 4 shows the staining pattern for the cohort. In all, 70% (36 out of 51) of cases showed high intensity of staining for c-Myc protein, while 85% (29 out of 34) of cases with detectable staining had more than 76% positive cells, also considered as high expression. To verify the staining specificity, serial sections from 10 tumour specimens that were positive for 9E10 antibody were also stained using the C19 rabbit polyclonal anti-c-Myc antibody. Results revealed a staining pattern similar to 9E10. However, the staining intensity with C19 was weaker than 9E10. The specificity of these two antibodies was verified by Western blots in previous studies (Persons *et al*, 1997; Liao *et al*, 2000b). Figure 2 shows results of c-Myc *in situ* hybridisation and immunohistochemistry studies on samples considered to demonstrate low, moderate and high levels of c-Myc expression. Analysis of c-Myc protein localisation results in the nucleus or cytoplasmic compartments of normal and invasive cells within the tumours revealed that nuclear staining was positive in 41% of normal cells, compared to 22% of invasive cells (statistical significance at $P=0.01$ by McNemar's two-sided χ^2 test). The increase in relative cytoplasmic localisation of c-Myc protein, comparing normal (53.7%), to invasive cells (61.1%) was not significantly different. Thus, the data are consistent with partial exclusion of c-Myc from the nuclei of invasive breast cancer cells.

The FISH score was significantly associated with the percentage positivity of invasive cells, as seen on IHC studies of c-Myc. However, 40% of tumours displayed a low index of c-*myc* gene amplification, but still expressed high levels of c-Myc protein (Table 6), indicating the possibility of other mechanisms of over expression unrelated to gene amplification in at least some tumours. The FISH score was not significantly associated with the intensity of IHC staining in the invasive cells (not shown), in contrast to the IHC percentage positivity score.

Table 2 c-myc mRNA *in situ* hybridisation (ISH) results

Staining intensity	0	1	2	3	Percent positivity	1	2	3	4
Number of tumour samples in each category $N=51$	1	6	25	19	Number of tumour samples in each level category $N=51$	1	3	5	42

In all, 51 human high-grade breast carcinomas were analysed to determine the relationships between c-Myc mRNA expression and *c-myc* gene *in situ* hybridisation results. Data are shown in two ways in the above table. First, overall staining intensity of c-Myc-positive cells was scored as 0, 1, 2, 3 (low to high), and the number of tumour samples at each level of staining indicated on the line below. Next, the percentage of tumour cells staining was scored as 0, 1, 2, 3, 4 (low to high %, as discussed in Materials and Methods). The number of tumours at each level of percent cell positivity for c-Myc is then indicated on the line below.

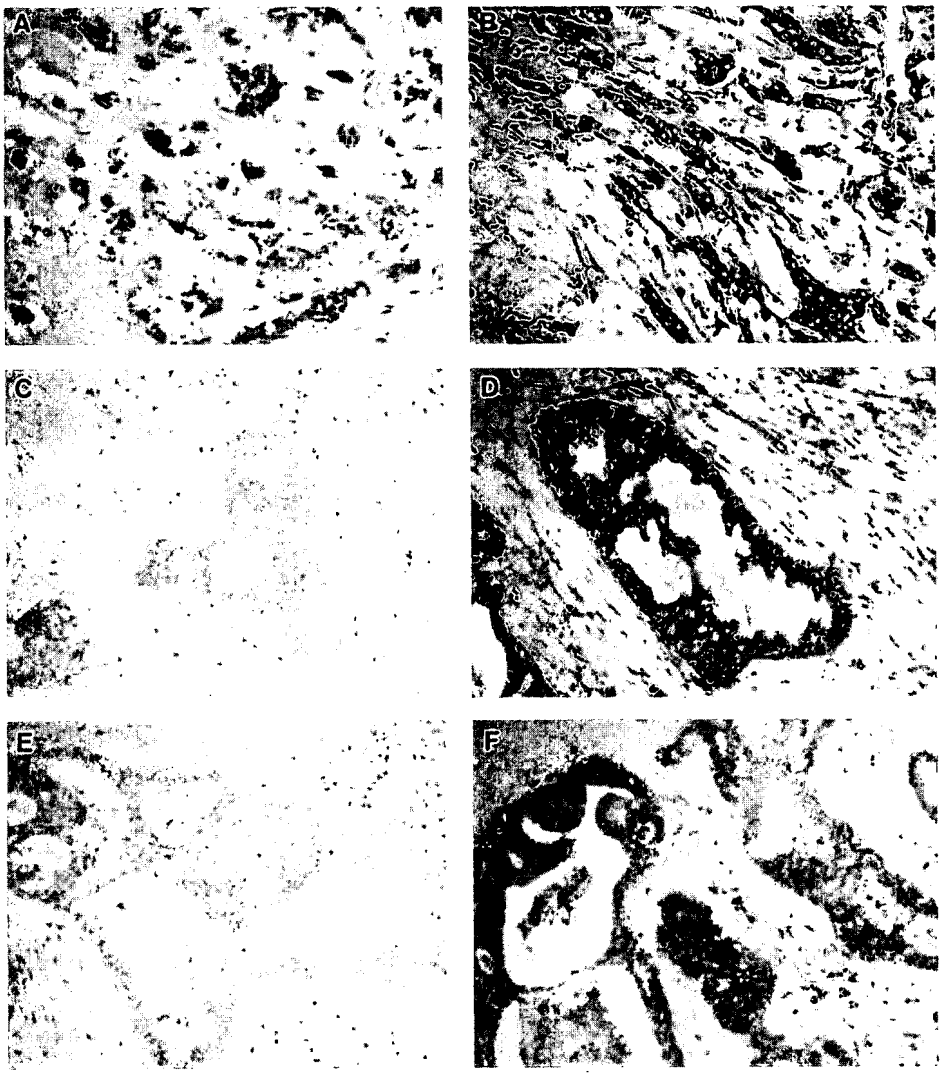


Figure 2 Immunohistochemical staining and *in situ* hybridisation for c-Myc of three sets of invasive ductal carcinoma. (**A**, **C** and **E**) High (3+), intermediate (2+) and low (1+) level of staining by immunohistochemistry for c-Myc. (**B**, **D** and **F**) High (3+), intermediate (2+) and low (1+) level of staining by *in situ* hybridisation.

DISCUSSION

Although there have been many reports on *c-myc* amplification in human breast cancer (Liao and Dickson, 2000), there are only two published studies involving application of the FISH technique to unfixed, frozen sections (Persons *et al*, 1997; Visscher *et al*, 1997), and one prior study using FISH on an archival human tissue microarray (Schraml *et al*, 1999). Another recent study applied FISH to evaluate *c-myc* amplification in ductal carcinoma *in situ* (DCIS) (Aulmann *et al*, 2002). Using the FISH technique on formalin-fixed, paraffin-embedded sections, we now show that 70% of high-grade breast cancer samples bear *c-myc* gene copy amplifications. Interestingly, the above-mentioned study, using FISH and focusing on DCIS, detected amplification of *c-myc* in only 20% of cases, but found a correlation of *c-myc* with increased tumour size and proliferation (Aulmann *et al*, 2002).

The level of amplification of *c-myc* in our study ranged between one and four additional copies of the gene; the majority (84%) of

the cases with the gene amplification gained only one to two copies, also consistent with FISH data reported for c-*myc* copy amplification in human metastatic prostate carcinoma tissues (Jenkins *et al*, 1997). The relationship between the level of c-*myc* gene copy amplification and the level its increased mRNA expression has been examined previously in breast cancer cell lines (Hyman *et al*, 2002). In general, it has been concluded that the two scores coordinate for c-*myc*, as is the case for many breast cancer genes. However, only 44% of the highly amplified genes, in general, showed increased RNA expression, and only 10.5% of the highly overexpressed genes were gene copy-amplified in the cell line study (Hyman *et al*, 2002). Another analysis was conducted to study of relationships between gene amplification and expression of 6095 genes in 37 intermediate grade human breast tumours. This study demonstrated that 62% of the highly amplified genes also showed elevated expression; overall, a two-fold change in DNA copy number was associated with a 1.5-fold change in mRNA levels. Overall, 12% of the variation in gene expression in the breast tumours studied was associated with gene copy number variation (Pollack *et al*, 2002). Further study of additional human breast tumours, at precisely defined grades and stages, will be necessary in order to more fully define the relationships between DNA copy numbers and expression of genes. The studies we report here indicate higher levels of c-Myc gene amplification and expression, than other previous reports in breast cancer. We believe that this is probably the result of our analysis of individual tumour cells in a well-defined set of high-grade breast tumours. Prior c-Myc expression and amplification microarray studies used tumour specimens which contain normal stromal components, potentially underestimating amplification and expression levels of the invasive tumour components (Pollack *et al*, 2002).

Our study reports a percentage of tumours gene amplified for c-*myc* (using FISH in high-grade tumours) that is much higher than the average figure (15.5%) reported in the literature (Isola *et al*, 2002). Most of the prior studies have employed the relatively insensitive Southern blot technique, and were reviewed in a recent meta-analysis (Deming *et al*, 2000). Consistent with this prior literature background, a recent study of 94 lobular and ductal breast cancers assessed amplification of c-*myc* by using a semiquantitative PCR assay and protein expression, with

Table 3 Correlations between c-*myc* gene copy number (FISH) mRNA expression (ISH)

	FISH	
	Low	**High**
(A) ISH (% cells)		
Low	*1*	3
High	*19*	*18*
		$P=0.0067$
(B) ISH (intensity)		
Low	2	*5*
High	*18*	*16*
		$P=0.0006$

Serial sections of high-grade human breast carcinomas were scored for c-*myc* gene copy number (FISH, Table 1) and mRNA expression (ISH, Table 2). In (A), a positive correlation ($P=0.0067$) was observed between tumour samples with a high percentage of cells demonstrating mRNA expression and a high c-*myc* gene copy number. A score of 2 or higher was classified as high on ISH, and a score of median or greater was categorised as high on FISH. In (B), a positive correlation ($P=0.0006$) was shown between a high level of intensity for c-Myc RNA expression a high and c-*myc* gene copy number. Note that a pairwise comparison of FISH and ISH was not possible for all cases, due to incomplete overlap of cases analysed with each assay.

Table 5 Nuclear/cytoplasmic localisation of c-Myc comparing normal and invasive cells

Normal cells (frequency percent)	**Invasive cells (frequency percent)**		**Total**
(A) Nuclear localisation			
	–	+	
–	28	4	32
+	14	8	22
Total	42	12	54
(B) Cytoplasmic localisation			
–	12	13	25
+	9	20	21
Total	21	33	54

In all, 54 pairs (normal *vs* invasive) of tissues were analysed to answer the questions of (1) whether positivity of nuclear cells in normal tissues is different from that in invasive cells, and similarly (2) whether positivity of cytoplasmic cells in normal tissues is different from that in invasive cells. The data are summarised in the above contingency tables. In all, 22 normal cell specimens were positive for c-Myc staining (40.71%), compared to 12 specimens (22.2%) in invasive cells. The difference is statistically significant ($P=0.01$) by McNemar's χ^2 test (two-sided).

Table 6 Correlation between c-Myc protein expression (IHC) and c-*myc* gene copy number (FISH)

	FISH	
IHC (% cells)	**Low**	**High**
Low	3	0
High	10	15
		$P=0.0016$

Consecutive serial sections of high-grade human breast tumours were scored for c-*myc* gene copy number or protein expression, by immunohistochemistry (IHC). IHC scores were defined in the Materials and methods section. Data were analysed for correlations between the results. A highly significant correlation was observed between high c-Myc protein expression (IHC) between percent cells positive and high c-*myc* gene amplification (FISH). $P=0.0016$ from two-sided McNemar's test. Note that for 15 cases, no staining for c-Myc could be detected; these negative cases were not included in the correlation presented, above.

Table 4 c-Myc immunohistochemistry (IHC) results

Staining intensity	**0**	**1**	**2**	**3**	**Percent positivity**	**1**	**2**	**3**	**4**
Number of tumour samples in each category	15	13	20	3	Number of tumour samples in each category	2	2	1	29

In all, 51 high-grade human breast carcinomas were analysed to determine the relationships between c-Myc protein expression and c-*myc* gene *in situ* hybridisation results. Data are shown in two ways in the above table. First, overall staining intensity of c-Myc-positive cells was scored as 0, 1, 2, 3 (low to high), and the number of tumour samples at each level of staining is indicated on the line below. Next, in a random subset of these cases, the percentage of tumour cells staining was scored as 0, 1, 2, 3, 4 (low to high %, as discussed in Materials and methods). The number of tumours at each level of percent cell positivity for c-Myc is indicated on the line below.

densitometry, after Western blot. These data showed c-*myc* gene amplification in 21% of tumours (Jenkins *et al*, 1997), using assays not based on *in situ* discrimination of tumour *vs* nontumour cells. The lower frequency of c-*myc* in this prior study is in contrast with the data we present here, and could be the result of the higher sensitivity and precision of the FISH and immunohistochemical methods, as distinct from quantitative PCR and Western blot densitometry. In addition, the 70% of amplified tumours in our study is also much higher than the 12% reported by Schraml *et al* (1999), using a c-*myc* FISH test on a tissue microarray. This large difference may be because the arrays are prepared from cores of paraffin-embedded tissue, as small as 0.6 mm in diameter which may contain too few tumour cells for complete analysis of amplification of a gene, such as c-*myc*. c-*myc* is known to be quite heterogeneous in its gene amplification within individual tumours (in contrast to *HER2/neu*, for example) (Persons *et al*, 1997).

Most previous reports on the expression of c-*myc* mRNA have utilised Northern blot, dot blot or PCR-based approaches, while just a few involved *in situ* hybridisation, which were primarily performed on frozen tissue sections (Liao and Dickson, 2000). Normal breast tissue is dominated by adipose cells, differing greatly from tumour tissue in its epithelial cellularity. Thus, normal and tumour tissues may not be rigorously compared by techniques involving RNA extraction from total tissue. Therefore, conclusions such as 'increased expression' may be more difficult to make from studies with Northern blot, dot blot and PCR-based techniques that require RNA extraction from tissues that have not been fastidiously micro-dissected for selection of tumour cells. Using a more sensitive, nonradioactive *in situ* hybridisation (ISH) approach on formalin-fixed, paraffin-embedded sections, we report herein high expression of c-*myc* mRNA in 92% of high-grade breast carcinomas. This figure is much higher than the recently reported data (22%), obtained by using a real-time RT–PCR method (Bieche *et al*, 1999). Dilution of the RNA from epithelium by the RNA from adipose in normal breast tissue in this latest prior report may be one of the possible explanations for this large difference.

In conclusion, the present study shows that approximately 70, 92 and 70% of biopsies of untreated high-grade breast cancer exhibit c-*myc* gene amplification, mRNA overexpression and protein overexpression, respectively. In most cases (84%), with gene copy amplification, the c-*myc* gene gains one to two additional copies. c-*myc* gene amplification was significantly associated with expression of its mRNA (both by intensity in invasive cells and by percentage positivity in invasive cells), and with expression of its protein (by percentage positivity in invasive cells). However, our data were also consistent with the prior literature on c-Myc (reviewed in Nass and Dickson, 1997; Liao and Dickson, 2000), indicating complex transcriptional, post transcriptional, translational and post-translational control of c-Myc expression *in vitro*. Specifically, in Table 5 we observed that in 40% of the high-grade tumours tested, c-Myc protein was expressed at high levels, despite a lack of its gene amplification.

It will be interesting to analyse lower grade tumours and premalignant lesions, with the same measurement tools, to determine if this c-*myc* amplification pattern is different, comparing different steps in onset and progression of the disease. Specifically, prior studies in fibroblasts and in human mammary epithelial cells (Liao *et al*, 1998, 2000a, b) have demonstrated that only a subtle deregulation of expression of c-Myc is sufficient to allow genomic instability. These prior cell biologic findings raise the question of whether c-Myc protein expression precedes or follows its gene amplification during the course of the natural history of breast cancer. It will also be interesting for future studies of lower grade breast cancers and premalignant lesions to determine whether there is evidence of nuclear exclusion of c-Myc protein. Indeed, nuclear exclusion of c-Myc in high-grade tumours could serve to attenuate its functions in later stages of disease progression (Liao and Dickson, 2000).

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